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LPS administration to the allergic nose contributes to lower airway inflammation

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Running head: A priming effect of allergen on nasal responsiveness to LPS
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Summery

Background Allergic rhinitis is an inflammatory reaction not confined to a single local compartment, but rather involving the whole airway system. Allergens known to induce allergic rhinitis are not always the sole trigger of the inflammatory reaction. Infections and organic dust might also cause exacerbations of rhinitis and associated conditions.

Objective To examine the effects of intranasal LPS exposure, as a surrogate for upper airway bacterial infections, in patients with symptomatic allergic rhinitis.

Methods 14 patients with a history of moderate to severe pollen induced allergic rhinitis were challenged intranasally with LPS. After 3 to 6 weeks the same patients were challenged again, first with allergen and 24 hours later with LPS. Nasal symptom scores, nasal lavage leukocyte counts and nasal airway resistance were assessed at 6 and 24 h after each provocation along with measurements of nitric oxide (NO) levels in nose and lung.

Results Six hours after the LPS challenge, increased level of leucocytes could be obtained in the lavage fluid, but no symptoms were experienced and no increase in nasal resistance could be recorded. The NO production in the upper and lower airways was similar before and 6 h after the provocation. In contrast, in patients exposed to pollen prior to the LPS challenge, both the nasal and the pulmonary NO levels were enhanced. This was accompanied by an increase in leukocytes.

Conclusion The present study demonstrates a priming effect of allergen on the nasal response to LPS as well as the presence of a systemic link between airway mucosal sites in upper and lower airways. This suggests that exogenously derived signals, like upper airway infections, can interfere with the initiation, maintenance and progression of asthma.

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Introduction

Allergic rhinitis is often associated with symptoms from the lung and has recently been recognized as a risk factor for the development of asthma [1]. It is suggested that aspiration of nasal secretion, nasobronchial reflexes and mediators transported via the circulation are important factors in linking the upper and lower airways [2, 3]. In accordance, intranasal corticosteroids reduce the bronchial hyperresponsiveness in asthmatic patients [4]. This has led to an operative definition of "allergic rhinobronchitis" [5] often referred to as "united airways disease" [6]. Allergens, known to induce allergic rhinitis, are not always the sole trigger of the inflammatory reaction. In fact, allergic rhinitis often occurs with concomitant exposure of dust and nasal viral infection. Although not as clearly linked to clinical manifestation of rhinitis as viral infections, there is support for the involvement of a secondary bacterial infection in the progress and severity of rhinitis since antibiotics are shown to be beneficial in the treatment of common cold [7]. However, the knowledge of the specific effects in the airways, induced by combined challenges with allergen and bacterial infection, is limited.

The upper airway is continuously exposed to micro-organisms and other foreign substances, like allergens. These are normally eliminated by the first layer of defence in the mucosa, consisting of mucus, ciliated epithelial cells and secretion of different molecules, such as lysozymes [8]. The recent finding of Toll-like receptors (TLRs) on epithelial cells has indicated a role for the nasal mucosa also in the primary immune response [9]. Crude LPS activates TLR2 and TLR4 and we have recently demonstrated that these receptors are up-regulated in the nasal mucosa of patients with symptomatic allergic rhinitis [10]. LPS inhalation results in a local increase of neutrophils and eosinophils as well as an increase of albumin and cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α [11, 12]. We have recently shown that nasal challenge with LPS down-regulates naturally occurring anti-inflammatory mediators [13] and this may influence other inflammatory reaction. Interestingly, high levels of LPS are detected in organic dust, which can affect the progress of the allergic rhinitis.

The present study was designed to evaluate if allergen could be an enhancer of non-specific inflammatory agents like LPS in the airways, focusing on pulmonary NO production, as a marker of airway inflammation, in patients with symptomatic allergic rhinitis.

Methods

Subjects

Fourteen non-smoking patients (9 women and 5 men) with a history of birch and/or grass pollen induced allergic rhinitis and 5 non-smoking healthy volunteers (3 women and 2 men) were included. The median (range) age of patients and controls was 27 (18–41) and 37 (25–58) years, respectively. The diagnosis of birch and grass pollen induced allergic rhinitis was based on a positive history of intermittent allergic rhinitis for at least 2 years and a positive skin prick test to birch and/or timothy pollen. All patients presented a wheal reaction diameter >3 mm towards birch or timothy in a skin prick test (roughly corresponding to a 3+ or 4+ reaction when compared with histamine) and they were classified as having moderate to severe symptoms during birch and/or grass pollen season. They had all during previous seasons been treated with oral antihistamines and nasal steroids. None of the patients used asthma medication on a regular basis (neither long acting β- agonists nor inhaled steroids) and they were free of upper and lower airway symptoms at the time of their first visit. Patients treated with local or systemic corticosteroids within 2 months before their first visit were excluded. Control subjects were symptom-free, had no history of allergic rhinitis and had a negative skin prick test to the standard panel of allergen. They were all free of medication. Before inclusion, all subjects, patients as well as controls, were evaluated by an ear-, nose- and throat consultant performing rhinoscopy. Individuals with a history of perennial symptoms or upper airway infection within 2 weeks before the first visit were excluded along with those exhibiting symptoms or signs of chronic rhinosinusitis, hypertrophy of turbinates, severe septum deviation or nasal polyposis. The study was approved by the Ethics Committee of the Medical Faculty, Lund University, and an oral and written informed consent was obtained from all participants.

Study design

Patients with allergic rhinitis were challenged intranasally with LPS. 100 µl of a sterile physiological saline solution with 0.25 µg/µl LPS (from E. coli, L-2654; Sigma, Saint Louis, Missouri, USA) was applied by intranasal spray to both nostrils after exsufflation. Three to 6 weeks later the same patients were exposed to birch or grass pollen (10,000 units in each nostril), followed by a LPS provocation 24 h later. The allergens used were specially selected in order to have an ultra-low LPS content (special delivery from ALK, Copenhagen, Denmark). The healthy controls were exposed to LPS alone using the same protocol. All challenges were performed before the start of the allergy season. One patient developed a

common cold during the assigned 3-6 week period between the two test occasions, and was therefore excluded from the second set of tests.

Nasal symptom scores were recorded 5 min, 30 min, 6 h and 24 h after each challenge. Nasal airway resistance was measured and nasal lavage fluid obtained at 6 and 24 h after each provocation along with measurements of nasal and pulmonary NO levels.

Skin prick test

Skin prick tests were performed with a standard panel of ten common airborne allergens (ALK, Copenhagen, Denmark) including pollen (birch, timothy and mugworth), house dust mites (*D. Pteronyssimus* and *D. Farinae*), molds (*Cladosporium* and *Alternaria*) and animal allergens (cat, dog and horse). It was carried out on the volar side of the forearm with saline buffer as negative and histamine chloride (10 mg/ml) as positive control. The diameter of the wheal reactions was measured after 20 min.

Symptom score

Subjects were asked to record the severity of three nasal symptoms, itching/sneezing, secretion and blockage using an arbitrary four-step scale from 0 to 3 (0=no, 1=mild, 2=moderate, 3=severe symptoms). A total nasal symptom score was calculated by addition of the three scores. A total lung symptom score based on cough, wheeze and dyspnoea were calculated by addition of the three scores. The combination of nasal and pulmonary scores is referred to as the total symptom score and it could maximally reach 18.

Nasal lavage

Nasal lavage fluid was obtained as previously described [14]; after clearing excess mucus by exsufflation, steril saline solution was aeorosolized into the nostrils and nasal fluids was passively collected in a test tube until 7 ml were recovered. The total number of cells/ml was counted in a Bürker chamber.

Rhinomanometry

Nasal airflow resistance was determined by active anterior rhinomanometry as previously described [15]. A pressure catheter was connected to one nostril. The flow was measured by a pneumotachograph (Rhinomanometer, gm-instruments, Kilwinning, UK) via an anesthetic mask. Each nasal passage was measured separately and a total resistance was calculated. The

recording of a pressure-flow curve was presented on a computer screen and total NAR was expressed as V_2tot representing the resistance of nasal airflow according to the polar co-ordinate system of Broms.

NO measurement

The levels of NO were measured by chemiluminescence with an NIOX® NO analyzer (Aerocrine AB, Solna, Sweden) and followed the "Recommendations for standardised procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide in adults and children" which has been compiled by the American Thoracic Society [16]. The NIOX analyser was calibrated weakly with a certified calibration gases (200 ppb for exhaled NO and 2000 ppb for nasal NO; Linde gas, Hoek Loos Speciality Gases, Amsterdam). The patients had not been engaged in any strenuous physical activity during the last 60 min prior to testing. During the measurements, air was continuously drawn into the analyzer at a constant sampling. All measurements were performed in a seated position. Fractional exhaled nitric oxide was sampled in exhaled air from the lungs via mouth using a tube. The nasal NO measurements were conduced at an aspiration flow rate of 5 ml/s using a nasal olive, in a way described in detail by Kharitonov and colleagues [17]. The ambient NO value was automatically subtracted from the NO measurements before the data were analysed. At each session three correctly executed measurements from upper and lower airways, respectively were recorded.

Statistics

All results are presented as means ± SEM and n equals the number of subjects involved. Statistical comparisons were made using Friedmans nonparametric test for repeated measurements with Dunns post test. *P*-values < 0.05 were considered statistically significant.

Results

Nasal challenge with LPS

A single dose of 50 µg LPS, applied by intranasal spray, induced no immediate symptoms in patients with a history of allergic rhinitis, and only 2 out of 14 reported a slight increase in nasal symptoms 6 h after the provocation (Fig. 1A). No symptoms or signs of systemic activation, like fever, headache, muscle pain, chills or lower respiratory tract symptoms, were obtained. The nasal resistance was not affected by the challenge (Fig. 1B), but an increased amount of leukocytes were found in all lavage fluids after 6 h: 2.7 ± 0.6 x 10^4 leukocytes/ml before and 8.8 ± 2.1 x 10^4 leukocytes/ml after the challenge (n=14; $p < 0.05$; Fig. 1C). Nasal and pulmonary NO levels was not affected by the challenge (Fig. D and E).

Healthy non-atopic volunteers exhibited a similar picture with no symptoms and signs of nasal or pulmonary engagement, but with a clear increase in NAL leukocyte recruitment: 2.7 ± 1.0 x 10^4 leukocytes/ml before and 7.9 ± 2.1 x 10^4 leukocytes/ml after the LPS challenge (n=5; $p < 0.05$). Nasal provocation with vehicle did not affect the leukocyte recruitment in patients and controls (data not shown).

Nasal challenge with LPS after allergen priming

Intranasally administered allergen (20,000 units) induced acute transient symptoms like sneezing, running, itching and blocked nose in all patients. These symptoms were most intense during the first half hour, but could still be recorded 6 h after the provocation. After 24 h no symptoms or signs remained (Fig. 2A). No lung affection was reported. The nasal airway resistance increased from 10.7 ± 1.8 V_2tot to 14.9 ± 2.5 V_2tot, without reaching significance (Fig. 2B). The resistance had returned to baseline after 24 h (10.2 ± 1.5 V_2tot). The number of leukocytes found in lavage fluid was not significantly increased after 6h (Fig. 2C; Note: no NAL was performed during the first hour). Nasal and pulmonary NO-levels remained unchanged (Fig. 2D and E). A differential count of neutrophils and eosinophils revealed that treatment with LPS alone induced recruitment of neutrophils whereas treatment with allergen induced recruitment of both eosinophils and neutrophils (Fig. 3).

Twenty-four hours after the allergen provocation all patients were challenged with LPS. No immediate symptoms were reported and no symptoms were recorded 6 and 24 h after the provocation (Fig 2A). The nasal resistance was not affected (Fig 2B), but an increased

number of leukocytes was found in the lavage fluid 6 h after the LPS provocation: 2.6 ± 0.6 x 10^4 leukocytes/ml before and 6.9 ± 2.5 x 10^4 leukocytes/ml after the challenge (n=12, $p< 0.05$) (Fig. 2C). An increase in nasal NO levels was noted 24 h after the LPS application, (705 ± 65 ppb and 811 ± 76 ppb before and 24 h after LPS, respectively (n=12; $p < 0.05$; Fig. 2D) and a marked increase in pulmonary NO was recorded both 6 and 24 h after the LPS provocation (27.7 ± 4.0 ppb, 32.7 ± 5.9 ppb and 34.6 ± 8.2 ppb before and 6h, and 24h, after LPS, respectively (n=12; $p < 0.05$) (Fig. 2E).

Discussion

Patients with a history of moderate to severe allergic rhinitis were intranasally challenged with LPS twice. The first time they were exposed to LPS alone, at the second time the LPS challenge was preceded by an allergen challenge. In both cases, the LPS application resulted in neutrophil recruitment. An increase in nasal and pulmonary NO production was only seen in patients primed with allergen. Healthy, non-atopic volunteers exposed to LPS alone, increased their amount of neutrophils in lavage fluid, but exhibited no increase in airway related NO production and no other symptoms or signs of airway affection.

Allergen applied in the nose in an immunologically sensitized patient results in a biphasic response, often referred to as early and late phase [18]. The early phase occurs within an hour and is caused by IgE-dependent activation of mast cells. A late phase reaction might occur within 4 to 8 hours and is often clinically indistinguishable from the early phase [19]. We have previously, using the same allergen provocation model as in the present study, demonstrated that a single dose of allergen, results in the development of marked symptoms (itching, sneezing and blockage) along with the recruitment of eosinophils and neutrophils [10, 20]. A similar effect on leukocyte recruitment was seen in the present study. Furthermore, LPS induced a sustained increase in the neutrophil recruitment both alone and after allergen priming. This is not unexpected since LPS is known to induce migration across the airway epithelium in murine models of lower airway diseases [21]. It is interesting to notice that an increased amount of neutrophils *per se* did not induce any nasal symptoms. This is in line with studies demonstrating that a moderate number of neutrophils always can be found in nasal smear from patients without symptoms [22].

Commercially available LPS is extracted from different strains of bacteria, but more than 90% of all publications have used LPS from enterobacteriaceae. According to a large survey by Dehus and co-workers, all the LPSs tested, regardless of the origin of microorganism (with the exception of LPS from P. aeruginosa and V. cholerae), were dependent on activation of TLR4 and were equipotent in the respect of cytokine release when incubated with whole blood cells [23]. The LPS used in the present paper was from E. coli (strain L-2654, Sigma) and the amount of 50 µg induced no symptoms or clinical signs of inflammation, with the exception of a marked increase of neutrophils in the lavage fluid. We have in a previous study, using the same concentration of LPS (from the same batch), in addition to the increase of neutrophils, reported enhanced levels of IL-6 and albumin as well as reduced levels of the

anti-inflammatory mediator uteroglobin. These findings reflect that an inflammatory process appears in the nose 6 h after the challenge [13]. A study using 40 µg LPS (E. coli 026:B6 Sigma) in healthy individuals also found an increase in nasal IL-6 together with histamine [25]. With 100-fold higher amount of LPS (5 mg; E. agglomerans), another study demonstrated the ability for LPS to enhance the incidence of sinusoidal symptoms and increase nasal levels of the inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα. The high dose used in this study was performed in workers that during daily basis already were exposed to organic dust, a complex mixture of microbes and compounds, known to contain large amounts of LPS [26]. An analogous increase of neutrophil infiltration together with an increase in nasal resistance with 20 µg LPS (E. coli O26:B6, Sigma) was found in a study on allergic children [24]. Despite differences in the LPS concentrations used for the nasal, challenge, all the cited studies showed enhancement of the local neutrophil and IL-6 concentrations without any accompanying signs of systemic reaction. In contrast, inhalations with LPS, in an amounts corresponding to the presently used, induced a systemic response with an increase in neutrophilia, CRP and temperature, in addition, a few subjects reported flu-like symptoms [27]. This difference might be related the fact the lung during normal conditions are next to sterile and the natural LPS exposure limited, whereas the nose is constantly exposed to different micro organisms as well as to low doses of LPS from different sources.

NO derived from the nose is implicated in the innate host defense against inhaled microbes [28] and LPS stimulation has been demonstrated to increase NO production *in vitro* [29]. It is well known that viral infections in the upper respiratory tract are associated with increases in the pulmonary NO production subsequently followed by exacerbations in patients with asthma [30-31]. The corresponding role of bacteria remains to be established and the information about changes in NO production following bacterial infections is limited [32]. It is therefore interesting to notice that nasal provocation with LPS alone did not affect the airway NO levels, whereas a clear up-regulation of the NO production was seen in patients primed with allergen. Thus, it appears that nasal exposure to LPS in combination with allergen has the ability to induce a subclinical pulmonary inflammation. This type of low grade inflammation is known to occur without any accompanying symptoms [33].

Nasal NO is increased in subjects with intermittent and persistent [34, 35] allergic rhinitis, and similar to NO increases in the lower airways, it responds to topical corticosteroid therapy [35,

36]. However, it is known that allergen challenge, by causing acute congestion, results in a drop in nasal NO levels, and these levels can remain decreased during several hours [37]. In line with this, no increase of NO was seen after allergen challenge. A limited increase in nasal NO could be obtained following the LPS provocation in allergen-primed patients. The difference of the NO kinetics between the upper and lower airways makes it important to stress that NO is a relatively poor predictor of local nasal inflammation [39]. Hence, it could not be excluded that the relatively small increase in nasal NO production seen in allergen primed patients following LPS application, reflects a substantial increase of the local inflammation.

Overproduction of NO has been implicated in the pathogenesis of airway inflammation in asthma and it has been shown that both exhaled NO and airway epithelial inducible NO synthase (iNOS) are increased in asthma [39]. A relation between pulmonary NO levels and the amount of infiltrating inflammatory cells, especially neutrophils and eosinophils, has been established [40, 41]. Hence, signs of increasing NO levels have been suggested to indicate pulmonary inflammation and to predict asthmatic exacerbations [42].

This study demonstrates a priming effect of allergen on nasal responsiveness to LPS. The results are supported by a study of Dubin and co-workers [43] demonstrating that allergen exposure increases the LPS responsiveness by the release of CD14 and lipopolysaccharide-binding protein into the airways. In analogy, a study in allergic children demonstrated that concomitant stimulation with LPS and allergen (*D. pteronyssinus*) enhance neutrophil infiltration [24]. Along the same lines, Michel and co-workers [44] showed that endotoxin might be an enhancer of the severity in asthmatics, sensitized and exposed to high level of allergen. Altogether this supports the idea of TLR4 as a factor that influences the allergic reaction. Further, the increase of pulmonary NO after nasal LPS application among allergen primed patients in the present study indicates a systemic link between airway mucosal sites in upper and lower airways. The mechanisms behind this remains to be clarified, but they might involve a systemic component of circulating or bone-marrow derived leukocytes, migrating into the airways, inducing a local inflammatory reaction [45-46]. Thus, a local nasal infection in allergen exposed immunologically sensitized patients might be involved in the initiation, maintenance and progression of asthma.

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Legends

Fig. 1. Patients with intermittent allergic rhinitis, before, 6 and 24 h after nasal LPS administration. A: Total symptom score representing the cumulative score from eyes, nose and lungs. B: Nasal airflow resistance determined by anterior rhinomanometry. C: Leucocytes in nasal lavage fluid. D: Pulmonary NO levels. E: Nasal NO levels. Results given as mean ± sem; n=14; *$p < 0.05$.

Fig. 2. Patients with intermittent allergic rhinitis, challenged with allergen followed by LPS 24 h later. Columns represent values before, 5 min, 30 min (A only), 6 h and 24 h after nasal allergen challenge as well as 6 and 24 h after nasal LPS administration.
A: Total symptom score representing the cumulative score from eyes, nose and lungs. B: Nasal airflow resistance determined by anterior rhinomanometry. C: Leucocytes in nasal lavage fluid. D: Pulmonary NO levels. E: Nasal NO levels. Results given as mean ± sem; n=12-13; *$p < 0.05$.

Fig. 3. Patients with intermittent allergic rhinitis, challenged with allergen followed by LPS 24 h later. A differential count of epithelial cells, neutrophils, eosinophils and mononuclear cells in nasal lavage fluid. Mononuclear cells represented less than 1% at all occasions). Columns depict the outcome of neutrophils and eosinophils. (n=12, *$p < 0.05$).

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